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# Differential In Vitro and In Vivo Antitumor Effects Mediated by Anti-CD40 and Anti-CD20 Monoclonal Antibodies Against Human B-Cell Lymphomas

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Summary: The antitumor effects of CD40 and CD20 monoclonal antibodies (mAbs) were compared on various human B-cell lymphomas by using both in vitro and in vivo assays. Anti-CD40 directly inhibited the proliferation of human B-cell lymphomas in vitro, whereas anti-CD20 exerted no inhibitory effects on the growth of any lymphoma tested. These lymphomas were then injected into immunodeficient mice to examine the antitumor efficacy of these unconjugated mAbs in vivo. This xenogeneic model was used in the evaluation of various potential therapeutic agents against human cancers in an in vivo setting. Surprisingly, in contrast to its negligible effects on lymphoma growth in vitro, anti-CD20 was more efficacious than anti-CD40 in promoting the survival of mice bearing some but not all lymphoma lines. To determine whether the antitumor effects of these mAbs were direct or indirect in vivo, we concurrently treated tumor-bearing mice with mAbs to the murine Fc receptor to block antibody-dependent cell-mediated cytotoxicity (ADCC). When these neutralizing antibodies against Fc receptors were administered at the same time as mAb treatment, the antitumor effects of anti-CD20 in vivo were completely abrogated, whereas anti-CD40 treatment, although also diminished, still provided significant antitumor effects. These results indicate that the in vivo antitumor activity of the murine anti-human CD20 mAb was primarily due to ADCC by murine effector cells, which may not translate into comparable effects in humans. By contrast, anti-CD40 may be of potential clinical use in the treatment of lymphomas in humans because of its additional direct antiproliferative effects. The results also demonstrate a possible difficulty in accurately evaluating the potential clinical efficacy of murine antibodies against human tumors in a human/mouse model system. Murine monoclonal antihuman antibodies may produce greater effects in human/mouse xenogeneic models, in which they are more likely to elicit host effector systems than when used in vivo in humans. Key Words: Anti-CD40—B Lymphoma—Anti-CD20—Human/mouse—Immunotherapy.

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In the treatment of cancer, the use of mAbs, either conjugated or unconjugated, offers a means of specifically targeting the tumor cell for destruction. However, the clinical use of unconjugated murine mAbs directed against cell-surface determinants for the treatment of B-cell lymphomas has met with mixed results. Several mAbs directed against

CD20, CD21, CD22, and CD24 surface determinants have been evaluated clinically in patients with various B-cell lymphomas (1,2). Although the toxicity was mild when compared with that of immunotoxins, the clinical responses that were observed were transient (2). Current preclinical evaluation of these anticancer agents involves the use of in vitro screening assays followed by determination of in vivo efficacy in tumor-bearing mice. The ability to engraft human tumors in immunodeficient mice has allowed for the evaluation of these and other reagents against human tumors in an in vivo setting (1,3). However, the real limitations of this model are still being elucidated.

CD40 is a member of the Fas family of molecules and is important in normal B-cell growth and differentiation (4,5). We have found that CD40-specific antibodies directly inhibit the growth of a variety of human B-cell lymphomas in vitro and are efficacious against human B-cell lymphomas in immunodeficient mice (6). Because CD20-specific mAbs have been previously evaluated clinically, we wanted to compare the effects of unconjugated anti-CD40 and anti-CD20 antibodies on B-cell lymphoma growth by using the human/mouse model. We report here that anti-CD40 is capable of directly inhibiting B-cell lymphoma growth in vitro, whereas antibodies to CD20 have no effect on tumor growth in vitro. However, anti-CD20 treatment was as efficacious as anti-CD40 in prolonging the survival of immunodeficient mice bearing human B-cell lymphomas, and these inhibitory effects were completely abrogated when 2.4G2 (antibodies against murine Fc receptors) were administered at the same time. The mechanism of the in vivo antitumor effects of anti-CD20 was presumably by host-mediated effects [i.e., antibody dependent cell-mediated cytotoxicity (ADCC) or complement-mediated lysis]. Therefore, these results indicate that whereas the use of murine antibodies may be efficacious in the treatment of human B-cell lymphomas, the human/mouse xenograft system may overestimate clinical efficacy of murine antibodies because these antibodies are better able to elicit host defense mechanisms in vivo in mice than in humans.

## MATERIALS AND METHODS

### Mice

C.B-17 *scid/scid* (SCID) mice were obtained from the Animal Production Facility (NCI-FCRDC, Frederick, MD, U.S.A.), and were not used until 6–8 weeks of age. SCID mice were kept under specific-pathogen-free conditions at all times. The mice were housed in microisolator cages, and all food, water, and bedding were autoclaved before use. SCID mice also received trimethoprim/sulfamethoxazole (40 mg trimethoprim and 200 mg sulfamethoxazole per 320 ml of drinking water) in suspension in their drinking water.

### Antibodies

Anti-human CD40 (M2 and M3 hybridomas, mouse IgG1) mAbs were kindly provided by Immunex (Seattle, WA, U.S.A.) (6). Anti-human CD20 (1F5, mouse IgG2a) mAb was a generous gift from Dr. Kevin Conlon (NCI-FCRDC; 7). Anti-human Fc receptor (2.4G2, rat IgG1) was purified ascites (8,9). Mouse IgG1 and IgG2a myeloma proteins were purchased from Cappel (West Chester, PA, U.S.A.).

### Tumor Cell Lines

The RL cell line was obtained from a patient with diffuse large-cell lymphoma (10). TU2C was an Epstein Barr virus (EBV)–induced B-cell lymphoma that was obtained from a SCID mouse that received human peripheral blood lymphocytes (huPBLs) from an EBV-seropositive donor. The generation of EBV-induced B-cell lymphomas has been previously demonstrated to occur in huPBL/SCID chimeras (11). The TU2C lymphoma is paucicional (unpublished observations) and has been kept in culture for ~1 year. Raji is a cell line cultured from a patient with Burkitt's lymphoma (12) and was a kind gift from Dr. John Ortaldo (NCI-FCRDC).

### Flow Cytometric Analysis of Cells

The protocol for flow-cytometric analysis has been described previously (13). Briefly, the cells were washed and counted by using a Coulter counter (Coulter Electronics, Hialeah, FL, U.S.A.). The cells were then adjusted to the appropriate concentration and were blocked with human AB serum to prevent nonspecific binding of immunoglobulin. The cells were then incubated with the appropriate primary antibody of either anti-CD40, anti-CD20, or an isotype-matched mouse IgG1 or IgG2a myeloma protein. The cells were then washed and incubated with a fluoresceinated

(FITC) secondary antibody; a goat–anti-mouse IgG (kindly provided by Dr. Kristin Komschlies (SAIC–Frederick). After washing, the cells were fixed in 1% paraformaldehyde and were analyzed on an EPICS flow cytometer (Coulter Electronics).

### Proliferation Assay

The effects of anti-CD40 and anti-CD20 on B-cell lymphoma growth in vitro was determined by [$^3$H]-thymidine incorporation (6). The cell lines were split 24 h before the assays were performed. In these experiments, the antibodies were cross-linked, which we have found to optimize the inhibitory signals (6). Briefly, 100 μl of goat–anti-mouse IgG (Fisher-Science, Pittsburgh, PA, U.S.A.) at a concentration of 25 μg/ml was added to the wells and incubated for 24 h at 37°C in 96-well, round-bottom, microtiter plates (Corning Glass Works, Corning, NY, U.S.A.). After washing with Hanks' balanced saline solution (HBSS) twice, the wells then received 100 μl of either anti-CD40, anti-CD20, or the isotype-matched IgG1 or IgG2a controls at 10 mg/ml and were allowed to incubate for 2 h. The tumor cells were then added at a concentration of $1 \times 10^5$ per well. Seventy-two hours later, 1 μCi of [$^3$H]-thymidine/well [sp. act, 6.7 Ci/mmol; (New England Nuclear Research Products, Boston, MA, U.S.A.)] was added for the final 8 to 18 h of culture. Cultures were harvested onto glass fiber filters with a PhD Cell Harvesting System (Cambridge Technology Inc., Cambridge, MA, U.S.A.), and [$^3$H]-thymidine uptake was assayed by liquid scintillation on an LKB betacounter (LKB Instruments, Inc., Turku, Finland). Each experiment was performed three to four times, with a representative experiment being shown. The data are presented as percentage growth compared with the isotype-matched control antibody. Cpm were analyzed, untransformed, by regression analysis and analysis of variance. When there was a significant departure from linearity, the difference between cpm levels for the control and treated groups at several concentration levels was tested for significance by Student's $t$ test by using the pooled residual error.

### In Vivo Experiments

All SCID mice received 20 μl of anti-asialo GM1 (aati-ASGM1) (Wako Chemicals, Dalias, TX, U.S.A.) i.v. 1 day before tumor transfer to remove host natural killer (NK) cells (13). Five million RL, TU2C, or Raji tumor cells were then injected either i.p. or i.v. SCID recipients then received 2 μg of either anti-CD40, anti-CD20, or the isotype-matched murine control antibody i.p. every other day for 20 days and a total of 10 injections starting on day 1, 3, or 7. For the experiments involving 2.4G2, 200 μg of either 2.4G2 or the isotype-matched rat control antibody was administered i.p. at the same time as the injections of anti-CD40 or anti-CD20 every other day for 10 days and a total of five injections. Tumor bearing mice were then monitored for tumor development and progression. Moribund mice were killed, and necropsies were performed on all mice for evidence of tumor. All experiments had five to seven mice per group and were performed two to three times. Both parametric (Student's $t$ test) and nonparametric (Wilcoxon rank sum test) analyses were performed to determine if the groups differed significantly ($p < 0.01$).

## RESULTS

### Surface Expression of CD20 and CD40 Molecules on Various Human B-Cell Lymphomas

We first examined the surface expression of CD20 and CD40 on various B-cell lymphoma cell lines derived from several types of lymphomas. RL is a cell line derived from a patient with diffuse large-cell lymphoma (10). TU2C is an EBV-induced B-cell lymphoma derived from an SCID mouse that received human peripheral blood lymphocytes from an EBV-seropositive donor. Raji is a cell line cultured from a patient with Burkitt's lymphoma (12). All of the lymphomas expressed comparable amounts of both CD20 and CD40, as determined by flow cytometry (Fig. 1). The CD40 expression on a Burkitt's lymphoma (Raji cell line) is in agreement with previous reports concerning these cell lines by our laboratory and others (6,14). It has been reported that CD20 expression is reduced in EBV lymphomas derived from huPBL-SCID mice (15). However, we failed to detect such a reduction in staining intensity in the TU2C EBV-lymphoma cell line. The flow-cytometric assessment therefore suggests that all of the cell lines examined expressed both these determinants.

### Anti-CD40 Directly Inhibits B-Cell Lymphoma Growth In Vitro

We then compared the effects of CD40-specific and CD20-specific mAbs on lymphoma growth in

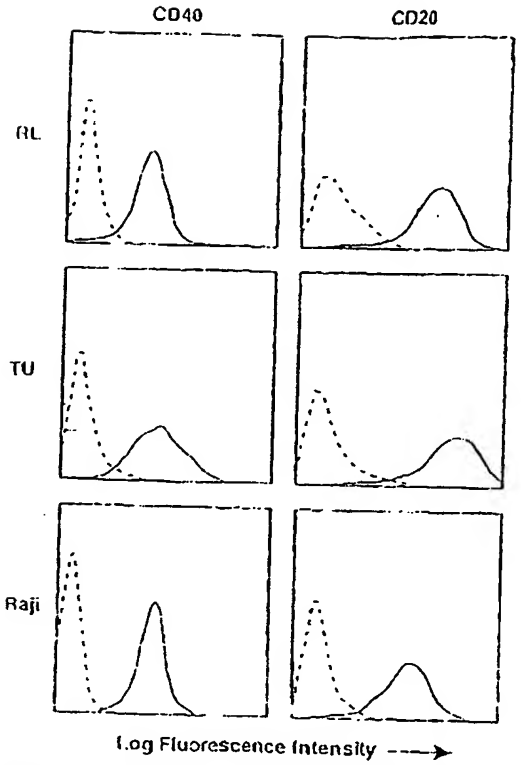


FIG. 1. Surface expression of CD40 and CD20 on human B-cell lymphomas as determined by flow-cytometric analysis. The flow-cytometric procedure has been described in Materials and Methods. The dotted lines represent control (msIgG) staining, and solid lines represent staining with either anti-CD40 or anti-CD20 mAb. Top: Staining of RL lymphoma cells with anti-CD40 (left panel) or anti-CD20 (right panel). Middle: Staining of TU2C lymphoma cells with anti-CD40 (left panel) or anti-CD20 (right panel). Bottom: Staining of Raji lymphoma cells with anti-CD40 (left panel) or anti-CD20 (right panel).

vitro by assessing the effects on [$^3$H]-thymidine incorporation. We previously found that anti-CD40 could directly inhibit B-cell lymphoma growth in vitro and that these inhibitory effects could be augmented by prior cross-linking of the antibody (6). Therefore, both antibodies (1 μg/ml) were cross-linked in these assays to produce optimal inhibitory signals. The data are presented as the percentage growth of the tumor cells compared with an isotype-matched control antibody. The results demonstrate that anti-CD40 is capable of directly inhibiting tumor growth of RL, TU2C, and Raji cell lines in vitro, whereas anti-CD20 produced no discernible effects on tumor proliferation, even after cross-linking (Fig. 2). The in vitro data would then suggest that, because of its ability to directly inhibit lymphoma growth, anti-CD40 mAb may be more efficacious in vivo when compared with anti-CD20 mAb, which produced no inhibitory effects in vitro.

### In Vivo Efficacy of Anti-CD40 and Anti-CD20 on Tumor-Bearing Mice

We then compared the efficacy of anti-CD40 and anti-CD20 on B-cell lymphoma progression in vivo. Anti-CD20 (1F5) has previously been examined clinically (2), and we wanted to evaluate its effects in a human/mouse tumor model in which human tumors are placed in immunodeficient SCID mice. This model has been used for the evaluation of other nonconjugated antibodies directed toward CD21, CD22, and CD24 on EBV-induced B-cell lymphomas placed in these mice (1). However, because these antibodies were of murine origin, there may be effector mechanisms active in SCID mice (NK cells, macrophages, complement) receiving these antibodies that may not predict human efficacy. RL tumor cells were injected i.p. into SCID mice, followed 1 day later by either anti-CD40 or anti-CD20 treatment (2 μg/day given every other day i.p. for 20 days). Treatment with anti-CD40 resulted in significant ($p < 0.05$) increases in survival in the SCID recipients (Fig. 3). This is in agreement with previous data from our laboratory concerning the antitumor properties of anti-CD40 in vivo (6). The results also demonstrate that in contrast to its negligible effects on lymphoma growth in vitro, treatment with anti-CD20 also significantly ($p < 0.05$) promoted the survival of the tumor-bearing recipients. The differences in survival between the two treatments were not statistically significant. When the EBV-induced B-cell lymphoma cell line, TU2C, was injected into SCID recipients, similar results of the two antibodies in promoting survival were obtained. However, with this lymphoma, anti-CD20 treatment was superior ($p < 0.05$) to anti-CD40 at increasing survival of the recipients when treatment was initiated at day 1 (Fig. 4). We then wanted to compare the efficacy of the two treatments on yet another type of lymphoma, this time administered i.v. The Burkitt's lymphoma cell line, Raji, was transferred into the SCID recipients by i.v. injection, and the mice were then given i.p. injections of anti-CD40 or anti-CD20 mAb (2 μg every other day for 20 days) 7 days after tumor injection. The data demonstrate that both anti-CD40 and anti-CD20 mAbs were capable of significantly ($p < 0.05$) increasing survival of the mice by a comparable ex-

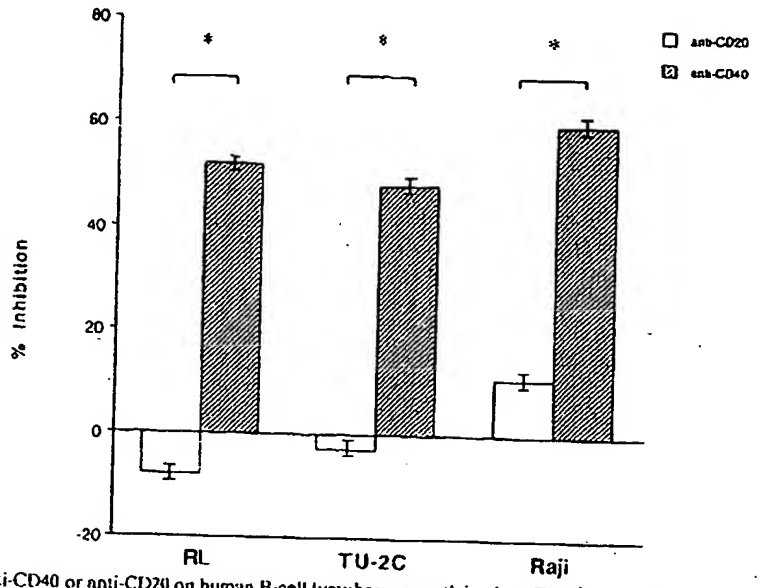


FIG. 2. Effects of anti-CD40 or anti-CD20 on human B-cell lymphoma growth in vitro. The [$^3$H]-thymidine-uptake proliferation assay is described in Materials and Methods. Data are presented as a percentage of growth inhibition compared with the isotype-matched control antibody. Representative of three to four experiments. *Values significantly ($p < 0.01$) less than those of anti-CD20–treated wells.

tent (Fig. 5). These results therefore demonstrate that both anti-CD40 and anti-CD20 are efficacious against a variety of human B-cell lymphomas in vivo, although only anti-CD40 demonstrated any direct antiproliferative effects on these tumors in vitro. In spite of its lack of effects in vitro, anti-CD20 was more efficacious than anti-CD40 toward the TU2C B lymphoma line.

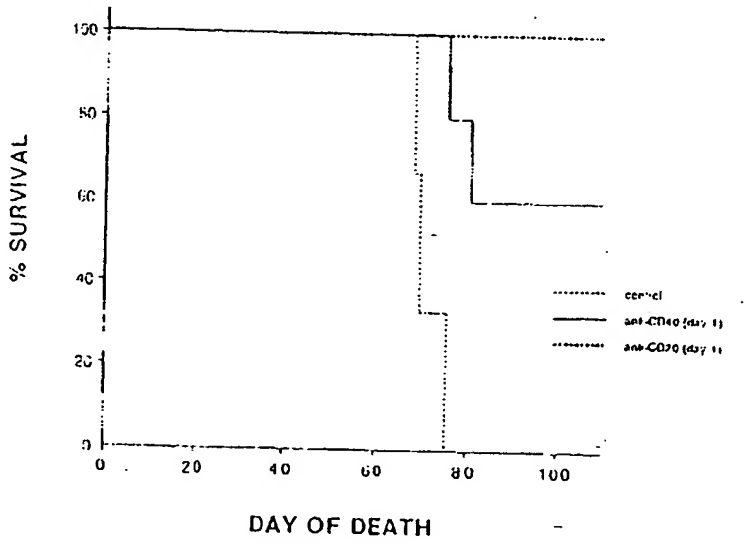


FIG. 3. Effects of anti-CD40 or anti-CD20 administration on the survival of SCID mice bearing RL tumor cells. The in vivo experiments are described in Materials and Methods. Tumor-bearing mice were treated 24 h after injection of tumor. Both anti-CD20 and anti-CD40 administration significantly ($p < 0.05$) promoted the survival of tumor-bearing mice. The differences in survival between the two treatments was not statistically significant.

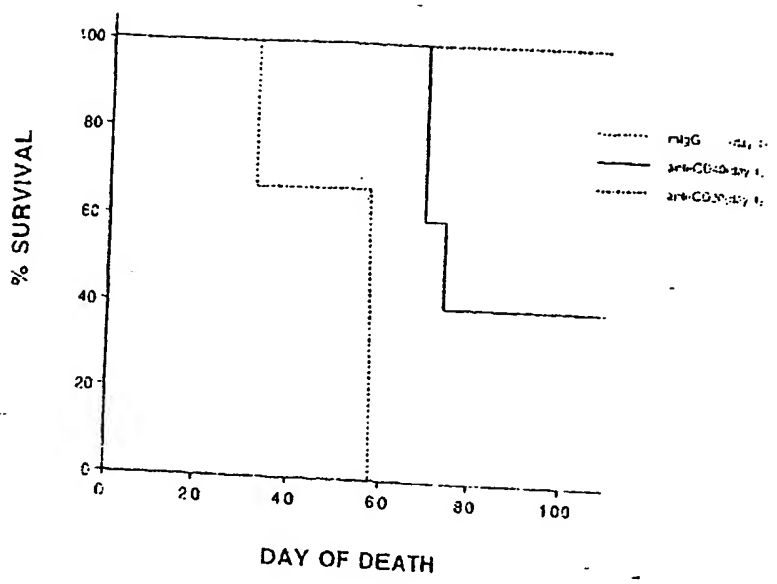


FIG. 4. Effects of anti-CD40 or anti-CD20 administration on the survival of SCID mice bearing TU2C tumor cells. In vivo experiments are described in Materials and Methods. Treatment was initiated on day 1. Both anti-CD40 and anti-CD20 significantly ($p < 0.05$) promoted the survival of SCID recipients. Anti-CD20 treatment was superior ($p < 0.05$) to anti-CD40 at increasing survival of the recipients.

### 2.4G2 Abrogates the Inhibitory Effects of Anti-CD20 in Tumor-Bearing Mice

We then determined the in vivo effects of anti-CD40 and anti-CD20 when the Fc receptors were blocked by 2.4G2, a mAb against murine Fc receptors (9). Anti-CD20 (1F5) is an IgG2a antibody, which is optimal for ADCC in mice (10). As shown in Fig. 6A and B, treatment with either anti-CD40 or anti-CD20 significantly ($p < 0.01$) promoted the survival of Raji-bearing SCID mice, with a significant percentage of the mice remaining disease-free. However, when 2.4G2 was administered at the time of mAb treatment, the effects of anti-CD20 were completely abrogated (Fig. 6A). Although 2.4G2 treatment also markedly reduced the antitumor effects of anti-CD40, anti-CD40 still significantly ($p < 0.05$) promoted the survival of Raji-bearing mice

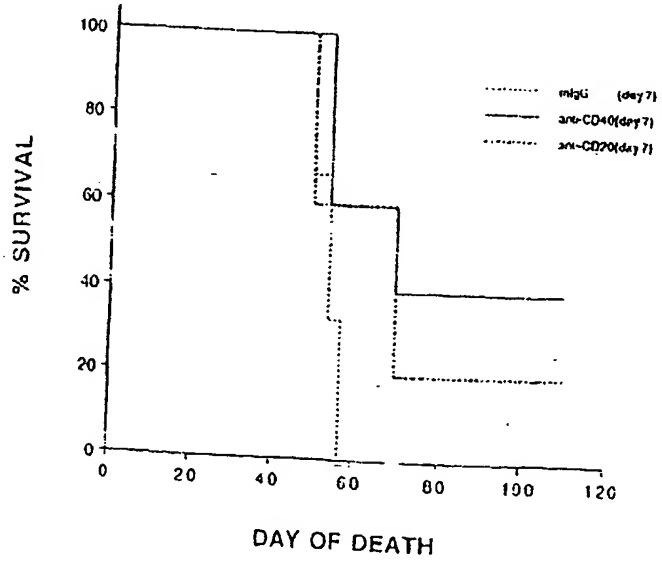


FIG. 5. Effects of anti CD40 or anti-CD20 administration on the survival of SCID mice bearing Raji tumor cells. In vivo experiments are described in Materials and Methods. Tumor-bearing mice were treated on day 7. Both anti-CD40 and anti-CD20 significantly ($p < 0.05$) promoted the survival of tumor-bearing mice. The differences in survival between the two treatments were not statistically significant.

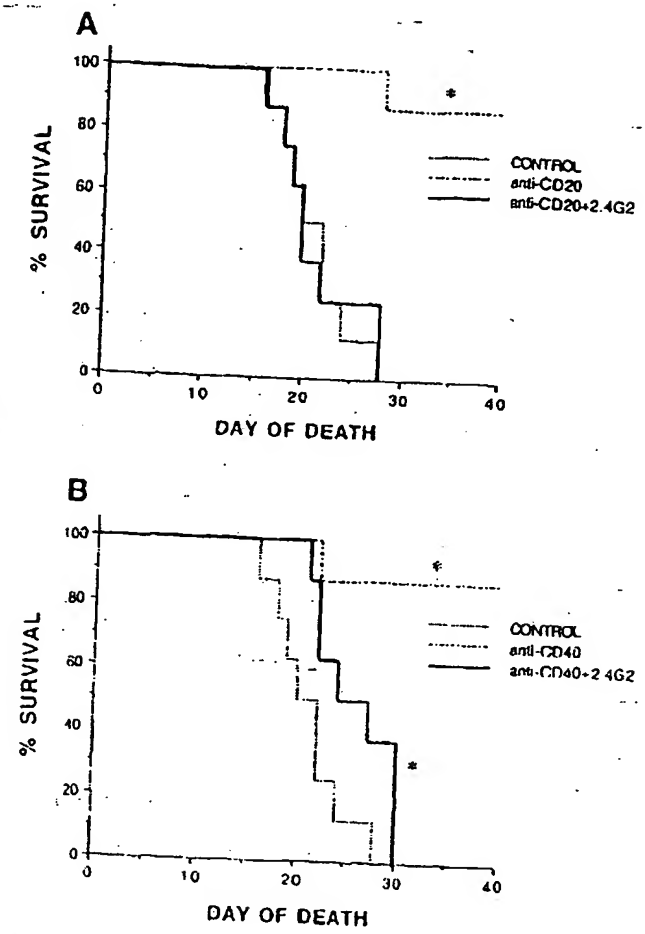


FIG. 6. Effects of 2.4G2 on in vivo antitumor effects of anti CD40 or anti-CD20 on SCID mice bearing RL tumor cells. In vivo experiments are described in Materials and Methods. When 2.4G2 (200 μg) was administered at the time of mAb treatment, the effects of anti-CD20 were completely abrogated (A). Although 2.4G2 also reduced the antitumor effects of anti-CD40, anti-CD40 still significantly ($p < 0.05$) promoted the survival of Raji-bearing mice (B). *Values significantly ($p < 0.05$) greater than those of control group.

(Fig. 6B). This indicates that the antitumor effects of anti-CD40 were not solely a function of the direct antiproliferative effects detected in vitro and were also caused by ADCC in vivo. However, the 2.4G2 data do suggest that the in vivo antitumor effects of anti-CD20 were exclusively by Fc receptor–mediated mechanisms, whereas anti-CD40 could still mediate significant antitumor effects in the absence of Fc-receptor function.

## DISCUSSION

We report here that anti-CD40 can directly inhibit the proliferation of several different human B-cell lymphomas in vitro, whereas anti-CD20 had no direct effects on tumor growth. However, both antibodies were similarly efficacious against these tumors in vivo. Because anti-CD20 had no effect on lymphoma growth in vitro, in contrast to the inhibitory effects of anti-CD40, we had initially attempted to use anti-CD20 as a control binding antibody in the xenogeneic tumor model to examine the efficacy of anti-CD40. The in vivo antitumor effects of anti-CD20 were as great as, or superior to, those of anti-CD40 in this xenogeneic model. However, the antitumor effects of anti-CD20 were completely abrogated when Fc receptors on host cells were blocked by 2.4G2. This indicated that there was little correlation between the in vitro inhibitory actions of anti-CD20 and efficacy of this antibody against these tumors in the human/mouse model. This would also suggest that using in vitro proliferation assays to screen potential anticancer mAbs is not sufficient because it is not possible to recreate

all of the potential mechanisms by which these antibodies could mediate antitumor effects in vivo.

The demonstration that anti-CD40 inhibits tumor growth in vitro may seem contradictory in light of previous reports stating that anti-CD40 had no effect on the growth of Burkitt's lymphoma cell lines (14). However, we found that prior cross-linking of the antibody optimizes the inhibitory signal by anti-CD40 in vitro (6), and this may be why the inhibitory effects of anti-CD40 were not detected in those studies, because only soluble antibodies were used. The data demonstrating that anti-CD20 did not affect lymphoma proliferation, even after cross-linking, are in agreement with those of previous reports (2) and suggest that anti-CD20 may be analogous to CD21, CD22, and CD24 antibodies, which also did not affect human B-cell lymphoma growth in vitro (1). In those studies, it was also found that nonconjugated anti-CD21, -CD22, and -CD24 antibodies could prolong survival in SCID mice bearing human EBV-induced B-cell lymphomas (1). The mAbs were also of murine origin and had an IgG2a isotype and subclass (1). IgG2a antibodies are the most efficient for ADCC, both in vitro and in vivo (16), and it appeared that the mechanism underlying the in vivo antitumor effects of these antibodies was by ADCC in the SCID recipients (1). The anti-CD20 mAb is also IgG2a, and it was speculated that the antitumor effects detected in some of the patients clinically were also mediated by ADCC and possibly complement fixation (2). However, the exact mechanism by which murine antibodies such as 1F5 mediate their modest antitumor effects clinically is not known (2). This would indicate that the use of a human/mouse model system for the evaluation of these murine mAbs may result in an overestimation of the degree of efficacy because of the increased ability of murine mAbs to mediate ADCC with murine effector cells, which would not necessarily be present in a clinical situation.

Additionally, a recent study comparing the efficacy of nonconjugated mAbs in nude and SCID mice-bearing human tumors found that nonconjugated mAbs were effective only in SCID mice, suggesting that the total absence of immunoglobulin in these mice heightens their ability to mediate ADCC when exogenous antibodies are administered (17). This would also indicate that the evaluation of murine mAbs of IgG2a subclass would yield excellent results (using SCID mice) that may be unlikely to translate into comparable clinical responses in humans. However, because the clinical evaluation of anti-CD20 suggested that its antitumor effects were due to ADCC, indicating that murine mAbs were capable of inducing ADCC with human effector cells, it is clear that evaluation by human trials is necessary to determine the efficacy and applicability of murine mAbs.

The anti-CD40 used in our studies was of an IgG1 subclass, which makes it difficult directly to compare the two antibodies in vivo. Experiments are under way evaluating anti-CD40 with another surface-binding mAb that is also an IgG1. However, differing surface expression of various surface antigens may also account for differing in vivo efficacy, making direct comparisons difficult (18).

Therefore, we are also attempting to create Fab' fragments of the CD40 and CD20 mAb to determine whether the antitumor effects persist both in vitro and in vivo. The data demonstrating that the superior efficacy of anti-CD20 diminished with increasing tumor burden suggest that the direct antiproliferative effects of anti-CD40 on neoplastic B-cells may offer additional advantages clinically. These results then suggest that the use of nonconjugated mAbs, particularly those with direct anti-proliferative effects on tumor cells, still have potential clinical applications. However, caution must be used in the interpretation of data generated by the use of a human/mouse model system in which effector mechanisms that are restricted to the mouse may lead to an overestimate of clinical efficacy.

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